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# Human CD4$^+$ T-Cell Response to Hepatitis Delta Virus: Identification of Multiple Epitopes and Characterization of T-Helper Cytokine Profiles

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**The T-cell-mediated immune response plays a crucial role in defense against hepatotropic viruses as well as in the pathogenesis of viral chronic hepatitides. However, very little is known about the role of specific T cells during hepatitis delta virus (HDV) infection in humans. In this study, the T-cell response to HDV in chronic hepatitis B virus (HBV) carriers with HDV superinfection was investigated at different levels. Analysis of peripheral blood mononuclear cell (PBMC) proliferation in response to a recombinant form of large hepatitis delta antigen (HDAg) revealed that 8 of 30 patients studied (27%) specifically responded to HDAg. By employing synthetic peptides spanning the entire HDAg sequence, we found that T-cell recognition was directed against different antigenic determinants, with patient-to-patient variation in the pattern of response to peptides. Interestingly, all responders had signs of inactive HDV-induced disease, while none of the patients with active disease and none of the control subjects showed any significant proliferation. More accurate information about the specific T-cell response was obtained at the clonal level. A panel of HDAg-specific CD4$^+$ T-cell clones from three HDV-infected individuals and fine-specificity analysis revealed that the clones tested individually recognized four epitopes corresponding to amino acids (aa) 26 to 41, 50 to 65, 66 to 81, or 106 to 121 of HDAg sequence. The study of human leukocyte antigen (HLA) restriction revealed that peptides 50 to 65 and 106 to 121 were presented to specific T cells in association with multiple class II molecules. In addition, peptide 26 to 41 was efficiently generated after processing of HDAg through the endogenous processing pathway. Cytokine secretion analysis showed that all the CD4$^+$ T-cell clones assayed were able to produce high levels of gamma interferon (IFN-γ), belonging either to T helper-1 (Th1) or Th0 subsets and that some of them were cytotoxic in a specific assay. This study provides the first evidence that detection of a specific T-cell response to HDAg in the peripheral blood of individuals with hepatitis delta is related to the decrease of HDV-induced disease activity. The HDAg epitopes identified here and particularly those recognized by CD4$^+$ T cells in association with multiple major histocompatibility complex class II molecules may be potentially exploited for the preparation of a vaccine for prophylaxis and therapy of HDV infection.**

Hepatitis delta virus (HDV) is a defective small single-stranded RNA virus that has been recognized to be a major cause of either acute or chronic hepatitis in humans (14, 51, 56), and outbreaks of often fatal epidemics have been reported in several parts of the world including developed countries (45, 46). HDV produces a single protein (hepatitis delta antigen [HDAg]) existing as both a small (p24) and a large (p27) form of 24 and 27 kDa, respectively, differing only in the addition of 19 amino acids (aa) at the C terminus of the large form (16). The biological cycle of HDV requires the contemporary presence of hepatitis B virus (HBV), which provides the coat and allows virion assembly and infectivity. HDV infection may occur either simultaneously with hepatitis HBV infection (coinfection) or as superinfection of an HBV carrier (19, 50, 52). HDV may be cleared after acute infection, which generally occurs after coinfection with HBV when the HBV has also been eliminated by the host, or may persist chronically in the liver, as typically observed after superinfection of an HBV carrier; this often progresses rapidly to cirrhosis and hepatocellular carcinoma (25).

It has been recently reported that mice made transgenic for both small and large HDAg and expressing these proteins in the liver do not develop any form of liver disease, providing consistent evidence that HDV is not directly cytopathic (29). The finding that HBV carriers undergo a more progressive and severe course of liver disease after HDV superinfection, with the presence of a remarkable infiltration of liver tissue by mononuclear cells, suggests that liver damage in hepatitis delta may be primarily immune system mediated (35, 40). On the other hand, some observations indicate that a specific immune response may play a role in protection against HDV infection as well as in the pathogenesis of liver injury. In this regard, it has been reported that HDV reinfection in HBV carrier chimpanzees recovering from a past HDV infection was characterized by a significantly reduced HDV replication (41). This suggests that the first contact with HDV may be followed by a partial immunity that is able to control viral activity.

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Studies on specific humoral immune response had never provided convincing evidence of the protective role of anti-hepatitis delta antibodies (anti-HD), although these antibodies are commonly detected in infected subjects. Indirect evidence in favor of the protective role of T cells in HDV infection has been obtained from both animal models and human disease. It has been shown that woodchucks infected with woodchuck hepatitis virus are partially protected from subsequent challenge with HDV when immunized with recombinant forms of HDAg in the absence of any detectable humoral response (34). Furthermore, HDV-infected woodchucks show an increased level of viremia after treatment with cyclosporin A, a specific inhibitor of T-cell-mediated responses (33). The role of T cells in controlling HDV infection in humans is indirectly suggested by the observation that HDV viremia is highly enhanced in human immunodeficiency virus-infected patients who present with a greatly reduced number of circulating $CD4^+$ T cells (47). However, a definition of the antigen specificity and function of T cells in HDV infection is still incomplete.

It has been well established that virus-specific T cells (both $CD8^+$ and $CD4^+$) play a crucial role in either killing infected cells, inducing neutralizing antibodies, preventing virus spread, or clearing extracellular viral particles. $CD8^+$ cytotoxic T lymphocytes (CTL) recognize T-cell receptor endogenously synthesized viral antigens, which are processed into the cytosolic compartment of infected cells; the peptides derived as a result are thereafter translocated into the endoplasmic reticulum lumen, where they bind class I molecules for presentation to CTL (8, 37). CTL kill target cells by two independent mechanisms, both of which induce DNA fragmentation and apoptosis of target cells: a secretory mechanism that is perforin and granzyme mediated, and a nonsecretory mechanism mediated by the interaction between Fas receptor (FasR) on targets and Fas ligand (FasL) on CTL (12, 21, 32). In addition to $CD8^+$ CTL, virus-specific $CD4^+$ T helper (Th) lymphocytes participate in viral clearance (6, 32). Th cells are induced following recognition of soluble antigens, which are processed in endocytic compartments of professional antigen-presenting cells (APC) (dendritic cells and activated monocytes), where the derived peptides can bind class II molecules (27). Moreover, $CD4^+$ T cells have been shown to play an important role in viral infections, since they are able to kill infected cells through either perforin release or FasR/FasL interaction (1, 32).

Both human and mouse $CD4^+$ T cells can be categorized into three subsets, namely, Th1, Th2, and Th0, depending on the cytokines they secrete (39, 48). In particular, Th1 cells characteristically produce gamma interferon (IFN-$\gamma$), Th2 cells produce interleukin-4 (IL-4) and IL-5 but not IFN-$\gamma$, and Th0 cells produce all these cytokines. It has been clearly demonstrated that Th1 or Th0 cells particularly polarized toward high IFN-$\gamma$ production can also display cytotoxic activity (20, 24).

The present studies were initiated to ascertain the main functional features and fine specificity of HDAg-specific $CD4^+$ T cells isolated from peripheral blood mononuclear cells (PBMC) of chronic HBV carriers with HDV superinfection. First, we evaluated the HDAg-specific proliferative response of peripheral T cells from patients with HDV infection, analyzing the relationship between such a response and HDV-induced disease activity. Second, we generated a panel of HDAg-specific $CD4^+$ T-cell clones from some HDV-infected individuals, studied their fine specificity to identify immunogenic epitopes of HDAg that could be exploited for a vaccine, and defined their cytokine secretion profile and cytotoxic activity.

## MATERIALS AND METHODS

**Study population.** Thirty chronic HBV carriers with HDV superinfection lasting no more than 10 years and 10 healthy subjects were studied. Infection with HBV and HDV was assessed by standard assays. HDV disease was defined as inactive when aminotransferase (ALT) levels had been normal for >1 year before the study and circulating immunoglobulin M (IgM) anti-HD was undetectable. Conversely, patients considered to have active HDV disease had elevated levels of ALT in serum (>40 IU/liter for >1 year) and constantly positive IgM anti-HD. Based on the presence of inactive or active disease, patients were categorized into two groups, A and B, respectively (Table 1). All HDV-infected patients underwent liver biopsy before this study, and the histological status of the liver specimens was assessed according to the following classification: cirrhosis, chronic active hepatitis, chronic lobular hepatitis, chronic persistent hepatitis, and normal liver.

**Reagents.** Recombinant forms of both large (p27) and small (p24) HDAg were expressed in *Saccharomyces cerevisiae*, and the reconstituted proteins were at least 80% pure as judged by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Coomassie blue staining (Chiron Corp., Emeryville, Calif.). The term HDAg used in the text refers to the large form of this antigen.

A panel of 27 16-mer synthetic peptides with an 8-aa overlap corresponding to the HDAg sequence of HDV genotype I was kindly provided by Chiron Mimotopes (Clayton, Australia). Each peptide contained free N termini and free C termini and was purified by high-pressure liquid chromatography to >80% purity. Lyophilized peptides were reconstituted at 20 mg/ml in dimethyl sulfoxide and diluted to 1 mg/ml with phosphate-buffered saline.

Recombinant vaccinia viruses (VV) expressing HDAg (VV-HDAg) were used for infection of target cells. The HDAg gene was inserted into the pSC59 expression vector, and construction and selection of VV containing the HDAg genes at the *tk* locus were performed as previously described (22). Wild-type vaccinia virus (VV-WT) was used as a control.

Analysis of the T-cell phenotype was performed by flow cytometry with the following fluorescin-conjugated monoclonal antibodies (MAbs): OKT3 (anti-CD3; Ortho Diagnostics, Raritan, N.Y.), OKT4 (anti-CD4; Ortho), OKT8 (anti-CD8; Ortho) and WT31 (anti-TCR $\alpha/\beta$; Becton Dickinson, Mountain View, Calif.). Cells were labelled with MAbs on ice for 30 min, washed four times, and immediately analyzed on a FACScan (Becton Dickinson) equipped with a 15-mW air-cooled 488-nm argon-ion laser.

**PBMC separation and proliferation assay.** PBMC were isolated on Lymphoprep cushions (CSM; Organon Teknika, Amersham, N.C.), washed three times, and resuspended in RPMI 1640 (Gibco Laboratories, Grand Island, N.Y.) supplemented with 2 $\mu$M L-glutamine, 1% nonessential amino acids, 1 $\mu$M sodium pyruvate, 100 U of penicillin per ml, 100 $\mu$g of streptomycin per ml, and 5% human AB serum (complete medium [CM]). PBMC at $10^6$ cells/ml in CM were cultured in 96-well flat-bottom microplates (Falcon Labware, Oxnard, Calif.) in the presence or absence of HDAg (3 and 1 $\mu$g/ml) or individual HDAg synthetic peptides at 10 $\mu$g/ml in 0.2-ml triplicate wells. After 6 days of culture, the cells were labelled for 18 h with 1 $\mu$Ci of [$^3$H]thymidine (Amersham, Little Chalfont, United Kingdom), and the DNA-incorporated radioactivity was measured by liquid scintillation counting. Data were expressed either as mean counts per minute (cpm) of triplicate cultures or as the stimulation index (SI), calculated as the ratio between cpm obtained in the presence of antigen and cpm obtained without antigen. None of the PBMC responded to a recombinant control antigen expressed in yeast (data not shown).

**Generation of HDAg-specific $CD4^+$ T-cell clones and antigen-specific proliferation in response to soluble HDAg.** PBMC from patients 1, 5, and 7 were resuspended in CM and plated ($10^5$ cells per plate) onto 96-well flat-bottom plates in the presence of 3 $\mu$g of HDAg per ml. After 5 days, cultures containing growing cells were resuspended in CM supplemented with 10 U of IL-2 (Proleukin; Eurocetus, Emeryville, Calif.) per ml. After an additional 5 days of culture, 50 U of IL-2 per ml was added, and on day 15, the cells were tested in an antigen-specific proliferation assay. Cultures found specific for HDAg were then cloned as previously described (5, 42). Briefly, T-cell clones were generated by limiting dilution at 0.3 cell/well onto 60-well Terasaki plates (Falcon) in the presence of 1 $\mu$g of phytohemoagglutinin (Wellcome, Beckenham, United Kingdom) per ml, 100 U of human IL-2 per ml, and irradiated (3,000 rads) allogeneic feeder cells ($5 \times 10^5$ cells/ml). After 10 to 12 days, cell growth was examined with an inverted microscope and then assayed for specific proliferative responses to HDAg with autologous Epstein-Barr virus-immortalized B lymphoblastoid cell lines (B-LCL) as antigen-presenting cells (APC). Specific T-cell clones were subcloned at 0.3 cell/well, and all developing subclones retained their HDAg-specific proliferation (data not shown). Fine-specificity analysis was carried out with APC consisting of autologous B-LCL pulsed with individual peptides in a 3-day proliferation assay. The routine proliferation assay of T-cell clones was performed by incubating $5 \times 10^4$ T cells for 72 h in the presence of autologous B-LCL used as APC, pulsed or not pulsed with HDAg or HDAg-derived peptides for 6 h. At 18 h before harvesting the cultures, 1 $\mu$Ci of [$^3$H]thymidine was added and the radioactivity incorporated by the cells was measured by liquid scintillation counting.

**Endogenous HDAg presentation and HLA restriction of HDAg-specific T-cell clones.** Autologous B-LCL were infected with either VV-HDAg or VV-WT at a multiplicity of infection of 10. Infected cells were incubated for 2 h at 37°C,

TABLE 1. HDAg-specific proliferative response in HDV-infected patients with either inactive (group A) or active (group B) HDV-induced disease

| Group | Patient no. | MHC (DR/DQ/DP) | Age (yr)/sex | ALT[a] | Histologic diagnosis[b] | Anti-HD IgM titer | PBMC response (cpm × $10^3$)[c] to HDAg at: | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | 3 μg/ml | 1 μg/ml | 0 μg/ml |
| A | 1 | 7,11/2,3/5,x | 30/M | N | CLH | Absent | **24** | **19** | 3 |
| | 2 | 11,11/5,5/ND[d] | 32/M | N | CLH | Absent | **3** | **2** | 0.6 |
| | 3 | 7,7/2,2/ND | 56/F | N | Normal | Absent | **9** | **3** | 0.6 |
| | 4 | 11,13/2,3/ND | 34/M | N | CAH | Absent | 20 | 13 | 22 |
| | 5 | 1,11/3,5/ND | 30/F | N | CAH | Absent | **7** | 1 | 1 |
| | 6 | 2,7/6,2/ND | 39/M | N | CAH | Absent | 2 | 1 | 1 |
| | 7 | 10,13/5,6/ND | 30/M | N | CAH | Absent | **10** | **4** | 0.5 |
| | 8 | ND/ND/ND | 36/M | N | CAH | Absent | 0.9 | 0.8 | 0.6 |
| | 9 | ND/ND/ND | 40/F | N | CAH/C | Absent | 9 | 8 | 4 |
| | 10 | ND/ND/ND | 42/M | N | CAH/C | Absent | 27 | 25 | 18 |
| | 11 | ND/ND/ND | 40/M | N | CAH/C | Absent | 0.8 | 0.6 | 1.2 |
| | 12 | ND/ND/ND | 31/M | N | Normal | Absent | **4** | **3** | 1 |
| | 13 | 2,11/6,3/ND | 30/M | N | CAH | Absent | 2 | 1 | 1 |
| | 14 | 7,11/2,3/ND | 23/F | N | CAH | Absent | **14** | **7** | 0.7 |
| | 15 | 1,11/3,5/ND | 43/F | N | CAH | Absent | **25** | **18** | 4 |
| B | 16 | ND/ND/ND | 61/M | 2 | CAH | 1:10 | 4 | 3 | 5 |
| | 17 | 4,4/3,3/ND | 29/F | 2 | CAH | 1:10 | 0.2 | 0.2 | 0.2 |
| | 18 | ND/ND/ND | 35/F | 2 | CAH | 1:100 | 7 | 6 | 4 |
| | 19 | 1,11/3,5/ND | 32/M | 2 | CAH | 1:10 | 3 | 2 | 2 |
| | 20 | ND/ND/ND | 45/M | 3 | CAH/C | 1:10 | 2 | 1 | 1 |
| | 21 | ND/ND/ND | 48/M | 3 | CAH | 1:1,000 | 5 | 3 | 4 |
| | 22 | 2,7/5,3/ND | 32/M | 2 | CAH/C | 1:10 | 5 | 3 | 4 |
| | 23 | 4,12/3,3/ND | 33/M | 5 | ND | 1:10 | 3 | 2 | 2 |
| | 24 | ND/ND/ND | 50/M | 6 | CAH | 1:10 | 1 | 0.5 | 0.5 |
| | 25 | ND/ND/ND | 45/M | N | CAH/C | 1:100 | 1 | 1 | 0.8 |
| | 26 | ND/ND/ND | 17/M | 2 | CAH | 1:10 | 0.5 | 0.5 | 0.6 |
| | 27 | 7,11/2,3/ND | 66/M | 3 | CPH | 1:10 | 0.4 | 0.4 | 0.4 |
| | 28 | 7,11/3,3/ND | 45/M | 2 | CAH | 1:10 | 0.4 | 0.4 | 0.4 |
| | 29 | ND/ND/ND | 22/F | 3 | CAH | 1:1,000 | 3 | 2 | 2 |
| | 30 | 2,11/3,6/ND | 20/M | 2 | CAH/C | 1:100 | 2 | 2 | 2 |

[a] ALT levels in serum are expressed as fold above upper limits. N, normal range (5 to 40 IU/liter).
[b] CPH, chronic persistent hepatitis; CLH, chronic lobular hepatitis; CAH, chronic active hepatitis; C, cirrhosis.
[c] The boldface values indicate a proliferation at least threefold above the control.
[d] ND, not determined.

washed twice, and then incubated overnight at 37°C in 5% $CO_2$. On the next day, the cells were washed three times, irradiated at 7,000 rads, and used as APC in a 3-day proliferation assay. In some experiments, APC were treated with cloroquine (Sigma-Aldrich, S.r.l., Milan, Italy) or brefeldin A (Sigma-Aldrich). For cloroquine treatment, the APC were preincubated for 10 min in CM containing 80 μM cloroquine before antigen pulsing, and the same concentration of cloroquine was maintained throughout pulsing, including washes and for the proliferation assay. For brefeldin A treatment, APC were incubated at the time of antigen pulsing with 4 μM brefeldin A. Then APC were fixed 0.05% glutaraldehyde (Sigma) in PBS for 1 min. The reaction was stopped with 0.2 M glycine (Sigma) in PBS vol/vol for 10 min at room temperature.

For human leukocyte antigen (HLA)-restriction analysis, blocking experiments were performed with culture supernatants of hybridomas L243, B7.21, and SPVL3 containing anti-DR, anti-DP, and anti-DQ MAbs, respectively. Hybridoma culture supernatants were added to the 96-well plates of the proliferation assay at a final dilution of 1:3. T-cell clones were then tested against HDAg-pulsed autologous or partially HLA-matched B-LCL. The B-LCL used here were JVM (DRB1*1102), LBS (DPB1*1701), WS7 (DRB1*0701), SWEIG (DRB1*1101), HERLUFF (DRB1*1201), RML (DRB5*0202), TEM (DRB1*1401), LG2 (DRB1*0101), HHKB (DRB1*1301), and Raji (DRB1*1001).

**Cytotoxicity assay.** The cytolytic activity of T-cell clones was measured in a 6-h $^{51}Cr$ release assay. As targets, $^{51}Cr$-labeled autologous B-LCL, pulsed or not pulsed with recombinant HDAg for 4 h, were used. The test was performed in U-bottom 96-well plates (Falcon) containing 5,000 target cells with effector/target ratios of 20:1, 10:1, and 5:1. All assays were performed in duplicate. The percent cytotoxicity was determined from the formula 100 × (experimental release − spontaneous release)/(maximum release − spontaneous release). Maximum release was determined by lysis of targets with detergent (0.1% Triton X-100). Spontaneous release was measured in supernatants from target cells incubated without effector cells, and assays were excluded from analysis if the spontaneous release was >25% of the maximum release in all assays.

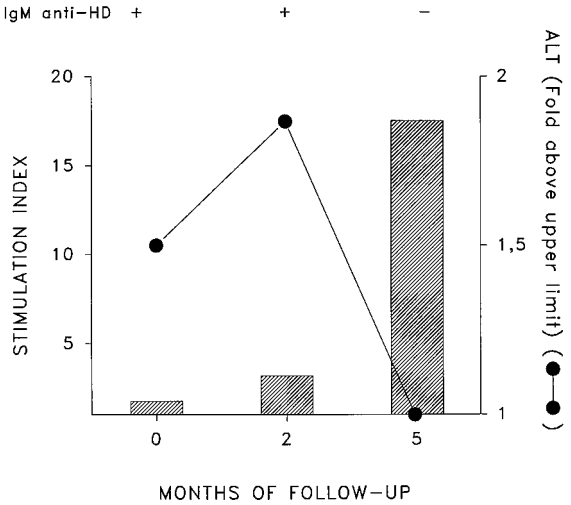


FIG. 1. Time course of HDAg-specific PBMC proliferation, ALT levels, and presence or absence of IgM anti-HD through 5 months of follow-up in patient 7. The proliferative response was assessed by [$^3$H]thymidine incorporation (hatched bars) and calculated as the SI. ALT is expressed as fold above the upper limit (40 IU/liter).

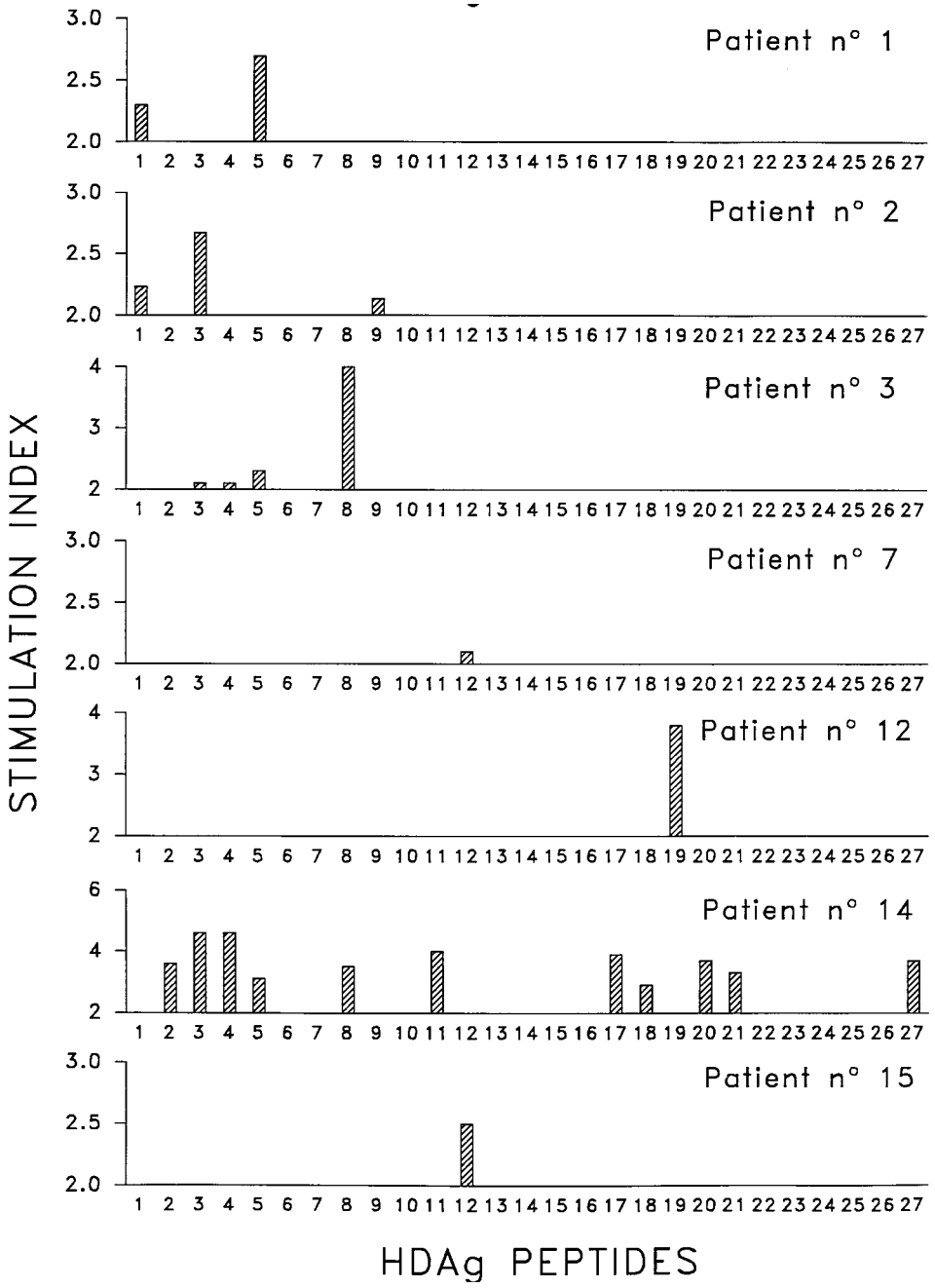


FIG. 2. Proliferative response of PBMC from patients, who responded to the whole HDAg, to a panel of synthetic peptides spanning the entire sequence of the antigen responded to at least one peptide. Specific proliferation was assessed by calculating [$^3$H]thymidine uptake after a 6-day culture and expressed as SI. Proliferations corresponding to a SI $> 2$ are shown.

**Cytokine production.** Cloned T cells ($10^5$) were cultured in 200 μl of CM in 96-well flat-bottom microtiter plates in the presence or absence of HDAg (9 mg/ml) presented by autologous B-LCL as APC ($10^5$) at 37°C. After 48 h of incubation, supernatants were collected and tested by sandwich enzyme-linked immunosorbent assay for the concentration of IL-4 and IL-5 (Pharmigen, San Diego, Calif.) and IFN-γ (23).

**Statistical analysis.** The difference between T-cell responses to HDAg for both patient groups was analyzed by contingency tables ($\chi^2$) with two degrees of confidence. A value of $P < 0.05$ was considered to be statistically significant.

## RESULTS

**PBMC proliferative response to recombinant HDAg and HDAg synthetic peptides.** PBMC isolated from HDV-infected patients were stimulated in vitro with recombinant HDAg. We found that PBMC from 8 of 30 patients (27%) significantly proliferated in response to antigen in a dose-dependent fashion (responders) (Table 1). Interestingly, all responders had inactive disease (group A). Conversely, PBMC from patients in group B (corresponding to patients with active disease) (Table 1) and from the healthy subjects tested (not shown) did not proliferate in response to HDAg. The difference between groups A and B was statistically significant ($P < 0.05$). In patient 7, determinations of T-cell proliferation, IgM anti-HD titers, and ALT levels were performed at different times. At 5 months after basal evaluation, a significant increase of specific

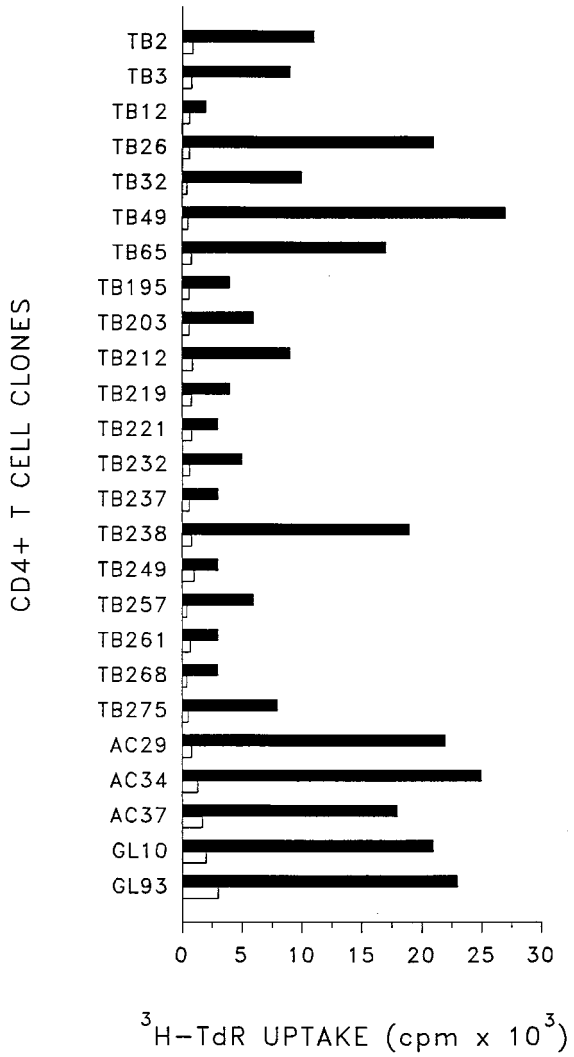


FIG. 3. HDAg-specific proliferative response of $CD4^+$ T-cell clones obtained from three chronic HBV carriers with HDV superinfection. TB clones were obtained from patient 1, AC clones were obtained from patient 7, and GL clones were obtained from patient 5. Specific proliferation was assessed after a 3-day incubation of cloned T cells in the presence of irradiated autologous B-LCL pulsed (solid bars) or not pulsed (open bars) with 9 μg of HDAg per ml. Data represent mean of triplicate determinations. $^3$H-TdR, [$^3$H]thymidine.

T-cell proliferation was observed simultaneously with the disappearance of circulating IgM anti-HD and normalization of ALT levels (Fig. 1).

To identify HDAg epitopes relevant for specific immune responses, PBMC from 18 patients (7 responders and 11 nonresponders to the whole HDAg) and 10 healthy subjects were tested against a panel of 16-mer overlapping peptides spanning the entire HDAg sequence. Proliferation was considered significant when the SI was >2. PBMC from all responders tested also proliferated in response to at least one HDAg peptide, with patient-to-patient variation in the pattern of response to peptides (Fig. 2). None of the PBMC from the nonresponders or healthy controls proliferated in response to any of the peptides tested (data not shown).

**Fine specificity and HLA restriction of HDAg-specific $CD4^+$ T-cell clones.** A panel of $CD4^+$ T-cell clones was established from HDAg-specific T-cell lines obtained from patients 1, 5, and 7. A total of 250, 230, and 270 clones were obtained from these three patients, respectively, and tested for their capacity to proliferate in response to autologous B-LCL pulsed or not pulsed with HDAg. As shown in Fig. 3, a total of 20 HDAg-specific clones were obtained from patient 1 (TB clones), 3 were obtained from patient 7 (AC clones), and 2 were obtained from patient 5 (GL clones). Blocking experiments performed on randomly selected T-cell clones demonstrated that the antigen-specific proliferation was independently blocked by either anti-DP, anti-DQ, or anti-DR MAb, indicating that HDAg can be presented in the context of all the class II gene products (Fig. 4). It was possible to ascertain, by the use of B-LCL lines of known HLA haplotype, that the restriction molecule for all nine DP-restricted TB clones tested was DPB1*1701. In contrast, the TB257 clone was DQB1*0201 restricted, while the TB203 and TB219 clones, as well as the GL10 and GL93 clones, were DRB1*1101 restricted. Interestingly, the TB238 clone recognized HDAg in the context of multiple DR molecules including DRB1*1101, DRB1*1102, DRB1*1201, DRB1*0101, DRB1*0701, DRB1*1401, and DRB5*0202. Finally, the AC29, AC34, and AC37 clones were DRB1*1001 restricted. Figure 5 shows the results of HLA restriction experiments on representative clones recognizing antigen in the context of the different alleles identified in this study.

Fine-specificity analysis revealed that all nine DP-restricted TB clones tested recognized the aa 26 to 41 peptide. Moreover, the TB238 clone recognizing HDAg in association with multiple HLA-DR molecules was specific for the aa 106 to 121 peptide, while the aa 50 to 65 peptide was presented by at least two different HLA-DR molecules, because it was recognized by either the DRB1*1101-restricted TB219, TB203, GL10, and GL93 clones or the DRB1*1001-restricted AC29, AC34, and AC37 clones. Finally, the DQB1*0201-restricted TB257 clone recognized the aa 66 to 81 peptide. Table 2 shows representative T-cell clones that individually recognized the different peptides identified in the present study.

**Recognition of endogenously synthesized HDAg and study of processing pathways.** The capacity of $CD4^+$ T-cell clones to recognize HDAg after processing through the endogenous pathway was investigated. T-cell clones that individually recognized all the HDAg epitopes identified in this study were selected and tested for their capacity to proliferate in response to APC previously infected either with VV-HDAg or VV-WT. We found that only the $CD4^+$ T-cell clones specific for the aa 26 to 41 HDAg epitope specifically responded to recombinant VV-HDAg-infected APC (Fig. 6). The finding that T-cell clones did not proliferate in response to APC, pulsed with supernatant collected after overnight incubation of B-LCL infected with VV-HDAg, rules out the possibility that HDAg was secreted by VV-HDAg-infected B-LCL and subsequently captured by APC and exogenously presented to T cells on class II molecules (results not shown).

It is known that cloroquine is an inhibitor of exogenous antigen processing by its ability to inactivate the endosomal-lysosomal proteases whereas brefeldin A is an inhibitor of protein synthesis that blocks the transport of newly synthesized major histocompatibility complex (MHC) molecules from the endoplasmic reticulum to the *trans*-Golgi (3, 11, 43). In antigen-processing experiments performed in randomly selected clones, preincubation of APC with either chloroquine or brefeldin A was found to inhibit the presentation of both endogenous and exogenous HDAg to specific T cells. Figure 7 shows the blocking effects by either brefeldin A or chloroquine on exogenous or endogenous HDAg presentation to the TB3 clone.

**Cytotoxic activity of HDAg-specific $CD4^+$ T-cell clones.** The capacity of HDAg-specific $CD4^+$ T-cell clones to lyse class $II^+$

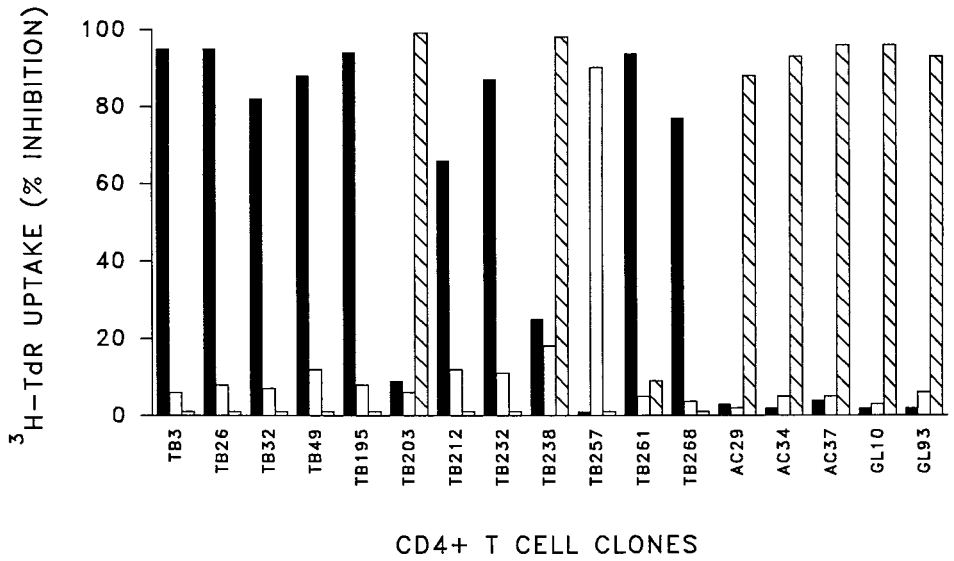


FIG. 4. Representative experiments of HLA restriction of HDAg-specific CD4$^+$ T-cell clones by using blocking anti-DP, -DQ, and -DR MAbs. T cells were incubated with HDAg-pulsed autologous B-LCL in the presence of anti-DP (solid bars), anti-DQ (open bars), and anti-DR (hatched bars) MAbs. Results were expressed as percent inhibition of specific T-cell proliferation by the following formula: percent inhibition = [1 − (cpm with MAb/cpm without MAb] × 100. Data represent means of triplicate determinations. $^3$H-TdR, [$^3$H]thymidine.

targets was investigated. We found that 3 of 20 T-cell clones from patient 1 (TB219, TB238, and TB261) and both clones from patient 5 (GL10 and GL93) killed autologous B-LCL cells used as targets that were previously pulsed with HDAg (Fig. 8).

**Cytokine secretion profile of HDAg-specific CD4$^+$ T-cell clones.** The functional analysis of T-cell clones was completed with the study of the cytokines secreted upon antigen-specific activation. T cells were tested for production of IFN-γ, IL-4, and IL-5 following stimulation with HDAg presented by autologous B-LCL. Cytokines were measured in the 48-h culture supernatant. This was the optimum timing as assessed in the course of preliminary tests. The assay was performed twice for each clone, with comparable results. All of the clones tested were able to produce cytokines only after stimulation with the specific antigen appropriately presented. The APC were not able to release any of the cytokines tested. Of the 19 clones tested, 7 produced only IFN-γ, therefore belonging to the Th1 subset, whereas all the remaining clones produced IFN-γ, IL-4, and IL-5 and thus belonged to the Th0 group (Table 3).

## DISCUSSION

In the last few years, several studies have greatly improved our knowledge of the structure of HDV and the molecular basis of its dependence on HBV for completion of its biological cycle (16, 56). However, although an immune system-mediated mechanism is believed to play a crucial role in both HBV clearance and hepatitis B pathogenesis (4–6, 18), the contribution of the immune system to the natural history of hepatitis delta has not been adequately investigated. During HDV infection, anti-HD antibodies of both IgM and IgG classes can be detected in the serum of infected individuals. In particular, a high-titer IgM anti-HD is strongly related both to elevated HDV viremia and to the severity of liver disease whereas isolated IgG anti-HD is found in patients with a more favorable course of HDV infection. However, the protective role of these antibodies is still under debate (9, 10, 49). On the other hand, the data so far available on the HDV-specific T-cell response are partial and inconclusive.

In this paper, we investigated for the first time the specificity and function of the HDV-specific CD4$^+$ T-cell response in HBV chronic carriers with HDV superinfection. First, we found that PBMC from 8 of the 30 patients studied (27%) specifically proliferated in vitro in response to HDAg. Interestingly, all eight of these patients (responders) had signs of inactive HDV-induced liver disease, as defined by the presence of both normal ALT levels and undetectable IgM anti-HD (Table 1). In contrast, no specific response was found in any of the patients with active HDV disease. A direct relationship between the presence of a significant HDAg-specific T-cell response and the decrease of HDV-induced disease activity is also suggested by noticing that in one patient with elevated ALT levels, detectable IgM anti-HD, and no HDAg-specific PBMC proliferation, a drastic increase of the specific peripheral T-cell response related to the disappearance of circulating IgM anti-HD and normalization of ALT levels was observed in course of follow-up (Fig. 1). Altogether, these results suggest that the appearance of an HDAg-specific T-cell response could be related to the down-regulation of HDV replication, possibly explaining the disappearance of IgM anti-HD with the consequent reduced activity of HDV-induced disease.

We also found that PBMC from all responders tested who were identified by the previous assay also proliferated to at least one HDAg peptide out of a panel of 16-aa overlapping peptides spanning the entire HDAg sequence (Fig. 2). The response to peptides was generally oligospecific, supporting the concept that the immune control of viral infections is usually established by the focusing T-cell response toward a restricted number of viral epitopes (60). The finding that an HDAg-specific response was detectable after only one in vitro stimulation and that PBMC from healthy subjects did not proliferate to HDAg and its peptides suggests that responding cells were presumably primed in vivo.

It can be reasonably argued that the HDAg-specific T-cell response was not detectable in patients with active disease

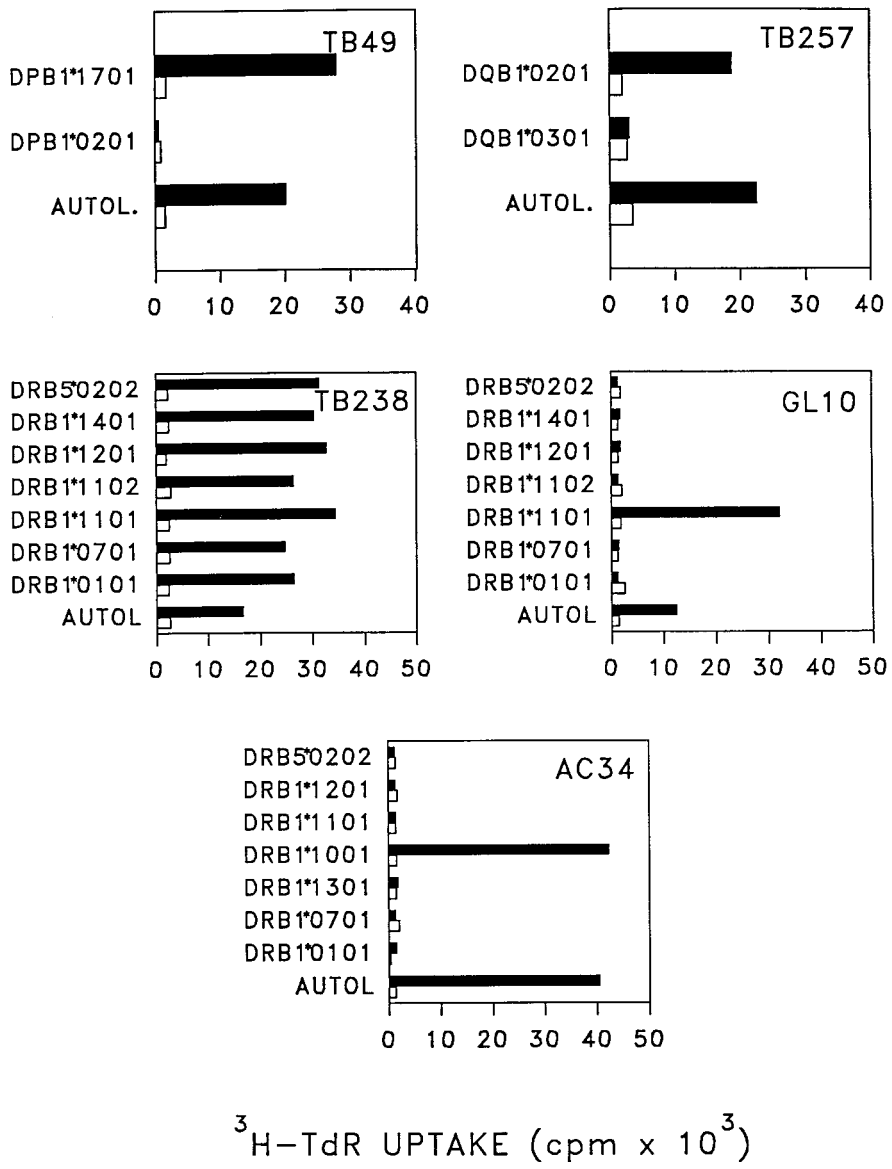


FIG. 5. Representative experiments of HLA class II restriction of HDAg-specific CD4+ T-cell clones. A series of homozygous B-LCL of known haplotype or autologous B-LCL were pulsed (solid bars) or not pulsed (open bars) with HDAg and used as APC. Data represent means of triplicate determinations. $^3$H-TdR, [$^3$H]thymidine.

because the specific T cells were mostly compartmentalized within the liver in the course of active inflammation, as suggested for other forms of chronic viral hepatitides (6, 38). If this is the case, HDAg-specific T cells would be present at a frequency too low to be detected in the peripheral blood compartment. Therefore, it could be of particular interest to study the Ag specificity of liver-infiltrating T cells in the course of chronic hepatitis delta.

In an attempt to characterize the function of HDAg-specific T cells, a panel of T-cell clones was generated from responder PBMC, previously stimulated with soluble HDAg. By this method, only antigen-specific CD4+ T-cell clones are usually obtained, since soluble antigens selectively gain access to the class II processing pathway (27). We found that all the HDAg-specific CD4+ T-cell clones analyzed produced large amounts of IFN-γ, belonging to either Th1 or Th0 subsets, and that some of them exerted cytotoxic activity in an antigen-specific fashion.

These HDAg-specific T cells could play a pivotal role either in defense against HDV infection or immunopathogenesis of liver disease. Indeed, IFN-γ could exert antiviral activities, either through direct suppression of viral replication, as recently demonstrated for HBV (29), or indirectly via its immunomodulatory activity, helping both professional APC capabilities and CD8+ CTL effector functions (53, 58). On the other hand, Th1 cytokines could amplify the liver damage, participating in nonspecific recruitment of memory T cells into the site of inflammation (54, 55). Furthermore, the large amounts of IFN-γ produced by these cells may contribute to the induction of class II molecules observed in the course of chronic hepatitides (26). In this connection, the finding that some of our CD4+ clones displayed antigen-specific cytotoxic activity

TABLE 2. Epitope mapping of five representative HDAg-specific CD4$^+$ T-cell clones with synthetic peptides

| HDAg peptide | SI of CD4$^+$ T-cell clones[a] | | | | |
|---|---|---|---|---|---|
| | TB3[b] (DPB1*1701) | TB238 (promiscuous) | TB257 (DQB1*0201) | GL93[c] (DRB1*1101) | AC37[d] (DRB1*1001) |
| 1–9 | 1.6 | 0.9 | 0.9 | 1.0 | 0.8 |
| 2–17 | 1.4 | 0.8 | 1.1 | 1.3 | 1.0 |
| 10–25 | 2.2 | 1.1 | 1.0 | 1.2 | 0.7 |
| 18–33 | 0.8 | 0.7 | 1.3 | 1.6 | 2.9 |
| 26–41 | **54.8** | 0.9 | 1.1 | 1.4 | 0.9 |
| 34–49 | 1.5 | 1.0 | 1.0 | 1.3 | 1.2 |
| 42–57 | 1.1 | 1.3 | 1.2 | 2.0 | 1.0 |
| 50–65 | 1.0 | 1.2 | 1.2 | **168.3** | **10.4** |
| 58–73 | 1.1 | 1.1 | 0.6 | 1.0 | 1.4 |
| 66–81 | 0.9 | 1.3 | **5.8** | 1.7 | 1.3 |
| 74–89 | 1.4 | 1.3 | 0.9 | 1.1 | 1.1 |
| 82–97 | 0.8 | 1.4 | 1.2 | 1.0 | 1.0 |
| 90–105 | 1.6 | 1.2 | 1.0 | 1.6 | 0.9 |
| 98–113 | 0.8 | 1.0 | 1.1 | 1.8 | 0.9 |
| 106–121 | 1.2 | **25.3** | 1.2 | 1.3 | 0.7 |
| 114–129 | 2.5 | 0.9 | 0.9 | 1.9 | 1.2 |
| 122–137 | 1.6 | 1.4 | 0.9 | 1.9 | 1.1 |
| 130–145 | 1.6 | 1.2 | 0.9 | 1.9 | 1.0 |
| 138–153 | 1.6 | 1.4 | 1.1 | 2.1 | 1.0 |
| 146–161 | 1.5 | 1.3 | 1.1 | 1.4 | 1.0 |
| 154–169 | 1.6 | 0.9 | 1.2 | 1.7 | 1.0 |
| 162–177 | 1.4 | 0.9 | 0.6 | 1.2 | 1.0 |
| 170–185 | 1.4 | 1.1 | 1.3 | 1.8 | 1.0 |
| 178–193 | 1.8 | 1.4 | 1.6 | 1.4 | 1.0 |
| 186–201 | 1.4 | 1.3 | 1.1 | 0.9 | 1.0 |
| 194–209 | 1.6 | 1.2 | 1.6 | 1.1 | 0.8 |
| 199–214 | 1.0 | 1.5 | 1.5 | 1.7 | 0.9 |

[a] Results are expressed as SI. Significant proliferations are shown in boldface type.
[b] TB3 is representative of TB26, TB32, TB49, TB65, TB95, TB212, TB195, TB232, TB249, TB261, and TB268 clones, all recognizing the aa 26 to 41 epitope in the context of the same restriction element (given in parentheses).
[c] GL93 is representative of the TB203, TB219, and GL10 clones, all recognizing the aa 50 to 65 epitope in the context of the same restriction element (in parentheses).
[d] AC37 is representative of the AC29 and AC34 clones, all recognizing the aa 50 to 65 epitope in the context of the same restriction element (in parentheses).

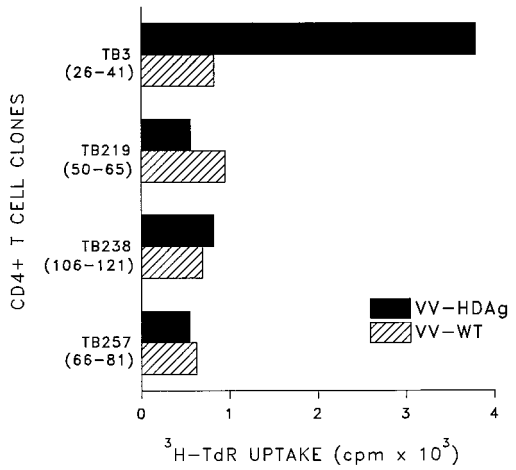


FIG. 6. Epitope 26-41 is efficiently generated after processing of endogenous HDAg for presentation to specific CD4$^+$ T cells. Autologous B-LCL were infected with either VV-HDAg or VV-WT at a multiplicity of infection of 10, incubated overnight, and then irradiated and used as APC in a 3-day proliferative assay in which T-cell clones with different fine specificities were used as effectors. Data represent means of triplicate determinations. $^3$H-TdR, [$^3$H]thymidine.

suggests that they could contribute to the lysis of hepatocytes, induced to express class II molecules by an inflammatory cytokine-rich milieu and presenting the appropriate viral peptides (6). It is possible that the lysis of a large number of uninfected class II$^+$ hepatocytes presenting the exogenous HDAg form causes severe liver necrosis and contributes to the severity of hepatitis. On the other hand, cytotoxic CD4$^+$ T cells may also contribute to viral clearance by the killing of infected hepatocytes expressing class II molecules. An increasing body of data shows that proteins synthesized intracellulary in APC may also associate with MHC class II and that they gain access to the MHC class II compartment through pathways that are incompletely characterized (31). In this regard, we found that CD4$^+$ T-cell clones specific to the aa 26 to 41 peptide recognized not only the exogenous form but also the endogenous form of HDAg, suggesting that this epitope is generated through both the exogenous and endogenous processing pathways. This indicates that some HDAg epitopes can be efficiently generated and presented to class II-restricted T cells by infected cells, which may be lysed in their turn by CD4$^+$ CTL.

The balance between the viral load (high versus low) and the strength of the host HDAg-specific T-cell response could be crucial for the preferential establishment of either immunity or immunopathology (13, 17, 59, 61). The mounting of a vigorous immune response could push this equilibrium toward the mechanisms inducing the infection resolution. Thus, in light of the data suggesting that HDAg-specific T cells could exert antiviral activities, it is tempting to speculate that the induction

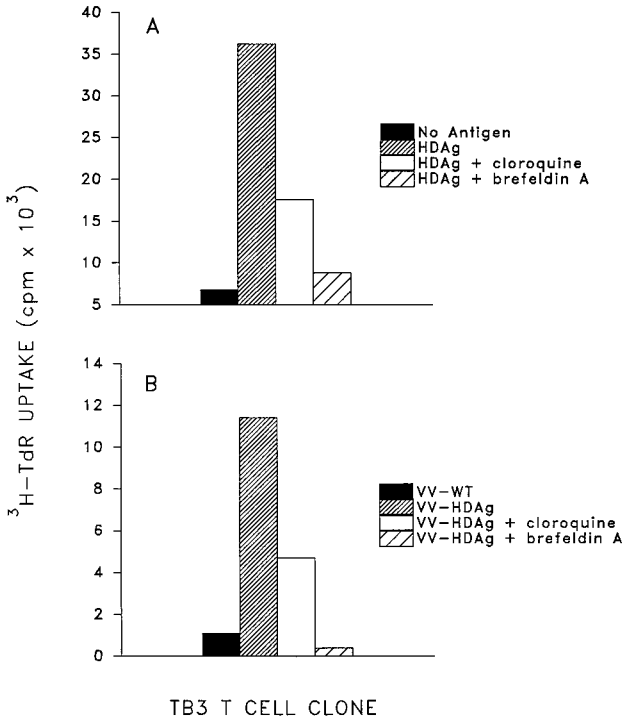


FIG. 7. Cloroquine and brefeldin A inhibit the processing of either the exogenous or the endogenous forms of HDAg for presentation to specific T cells. Autologous B-LCL pulsed with exogenous HDAg (A) or infected with VV-HDAg (B) were used as APC in the presence or absence of either cloroquine or brefeldin A. The TB3 clone, which recognizes both endogenous and exogenous HDAg, was used as the effector. Data are expressed as the mean of triplicate determinations. $^3$H-TdR, [$^3$H]thymidine.

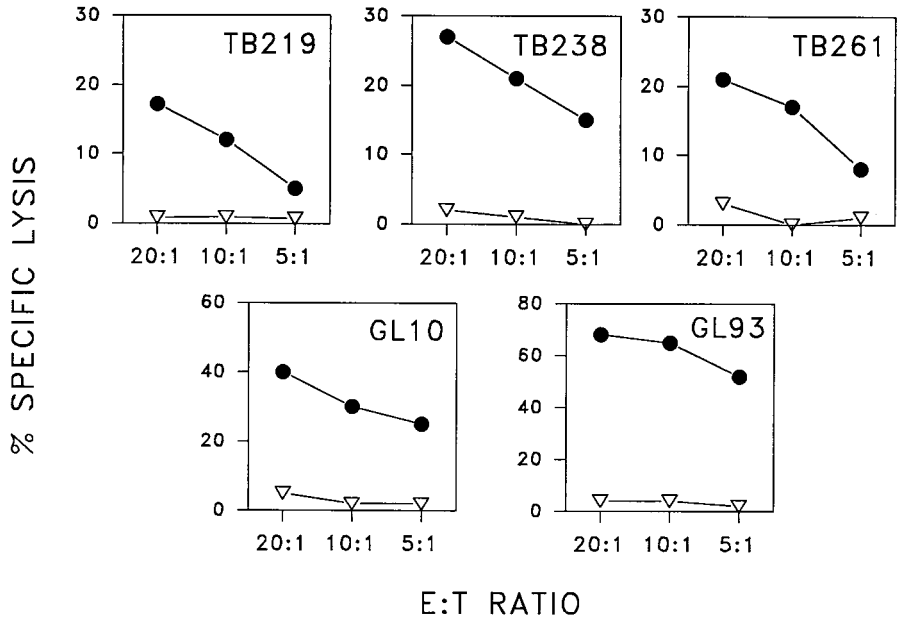


FIG. 8. HDAg-specific cytolytic activity of CD4+ T-cell clones derived from two HDV-infected individuals. Cytotoxicity was tested in a standard 4-h $^{51}Cr$ release assay with, as targets, autologous B-LCL pulsed (solid circles) or not pulsed (open triangles) with HDAg at different effector/target ratios as indicated. The percent specific lysis represents means of triplicate determinations.

and expansion of these cells by a vaccine containing HDV epitopes could be helpful for the prevention of HDV infection.

In this connection, the finding that HDAg was recognized in the context of different class II gene products (either DR, DP, or DQ alleles) may be crucial for induction of a polyspecific T-cell response and therefore of particular relevance for a vaccine design. Further, the fine-specificity study of our clones, carried out by using overlapping synthetic HDAg peptides, enabled us to identify four immunogenic peptides (aa 26 to 41, 50 to 65, 66 to 81, and 106 to 121), which could be potentially exploited as a vaccine preparation for several reasons.

First, we found that at least two peptides were recognized by T cells in association with more than one class II molecule. In particular, the aa 106 to 121 peptide was presented in association with multiple HLA-DR molecules (promiscuous peptides) (30, 44) and the aa 50 to 65 peptide was presented in association with two different HLA-DR molecules. The identification of these epitopes may be particularly relevant for preparation of peptide-based vaccines for the immunization of subjects with a different genetic background. Second, some of the T-cell HDAg epitopes identified are present within protein regions, which are critical for viral functions. In particular, the aa 26 to 41 epitope is part of a coiled-coil domain, the aa 66 to 81 epitope is included within the nuclear localization signal sequence, and the aa 106 to 121 epitope is included within the RNA binding domain (36). Thus, it is unlikely that mutations relevant to viral immune system escape can occur in regions encoding these T-cell epitopes, since they could significantly alter the HDV biological cycle. This evidence, together with the finding that HDAg shows a low degree of heterogeneity (15, 57), emphasizes the use of these sequences in a vaccine preparation.

Third, the four T-cell epitopes identified in this study largely correspond to sequences previously reported as also being recognizable by anti-HD antibodies in the serum of HDV-infected patients (9). Thus, these epitopes would satisfy a fundamental prerequisite for a vaccine, i.e., to be immunogenic for both the T- and B-cell compartments. Moreover, the evidence that a conspicuous number of our HDAg-specific T-cell clones displayed a Th0 profile suggests that they could promote not only cellular immune responses by the synthesis of Th1 cytokine but also efficient antigen-specific antibody responses by the production of Th2 cytokines.

Finally, the finding that the aa 26 to 41 epitope was gener-

TABLE 3. Cytokine secretion profile of HDAg-specific CD4+ T-cell clones[a]

| T-cell clone | Amt (pg/ml) of cytokine secreted | | | | | | Th subset |
|---|---|---|---|---|---|---|---|
| | IFN-γ | | IL-4 | | IL-5 | | |
| | Ag | No Ag | Ag | No Ag | Ag | No Ag | |
| TB3 | 4,316 | 0 | 963 | 0 | 123 | 0 | Th0 |
| TB32 | 1,934 | 0 | 2,066 | 0 | 1,040 | 0 | Th0 |
| TB49 | 6,062 | 0 | 1,511 | 0 | 770 | 0 | Th0 |
| TB65 | 6,254 | 0 | 100 | 0 | 3,483 | 0 | Th0 |
| TB195 | 5,208 | 0 | 1,472 | 0 | 1,383 | 0 | Th0 |
| TB203 | 6,188 | 0 | 1,808 | 0 | 843 | 0 | Th0 |
| TB219 | 6,416 | 0 | 1,860 | 0 | 376 | 0 | Th0 |
| TB232 | 4,724 | 0 | 1,987 | 0 | 1,521 | 0 | Th0 |
| TB237 | 3,892 | 0 | 168 | 0 | 172 | 0 | Th0 |
| TB238 | 2,790 | 0 | 1,445 | 0 | 565 | 0 | Th0 |
| TB249 | 5,814 | 0 | 1,024 | 0 | 1,031 | 0 | Th0 |
| TB257 | 6,474 | 0 | 123 | 0 | 3,240 | 0 | Th0 |
| TB261 | 2,534 | 0 | 0 | 0 | 0 | 0 | Th1 |
| TB268 | 6,952 | 0 | 0 | 0 | 0 | 0 | Th1 |
| GL10 | 1,808 | 0 | 0 | 0 | 0 | 0 | Th1 |
| GL93 | 1,704 | 0 | 0 | 0 | 0 | 0 | Th1 |
| AC29 | 2,114 | 0 | 0 | 0 | 0 | 0 | Th1 |
| AC34 | 1,780 | 0 | 0 | 0 | 0 | 0 | Th1 |
| AC37 | 1,808 | 0 | 0 | 0 | 0 | 0 | Th1 |

[a] Cytokine production was tested in supernatant from the indicated T-cell clones stimulated with autologous B-LCL pulsed or not pulsed with HDAg (9 μg/ml), as described in Materials and Methods.

ated during processing of both exogenous and endogenous HDAg suggests that it can be efficiently presented to class II-restricted T cells by infected cells, thus allowing control of viral spreading by the killing of infected hepatocytes. Both brefeldin A and cloroquine blocked the presentation of endogenous HDAg to specific $CD4^+$ T cells, confirming for the cytosolic proteins the existence of a cloroquine-sensitive endogenous processing pathway for their presentation on class II molecules (27). On the other hand, the finding that not all the epitopes of exogenous HDAg were generated after processing of the endogenous HDAg gaining access to the class II compartment indicates that the set of HDAg peptides generated from these two routes is different, as previously shown for other antigens (2).

In conclusion, in this study we have shown that detection of the HDAg-specific T-cell response in the peripheral blood of HDV-infected patients is related to a reduced activity of HDV infection. Analysis at the clonal level suggests that the development of HDAg-specific Th cells with a Th0 or Th1 phenotype and cytotoxic function may contribute to both amplification of liver damage and viral clearance. Moreover, the analysis of specific T-cell clones allowed us to identify five different HDAg immunogenic epitopes. These data provide new and important information on the T-cell immune response against HDV that could be exploited for developing a protective vaccine that could be used for prophylaxis and therapy of HDV infection.

## ACKNOWLEDGMENTS

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